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Targeted delivery of CCR2 antagonist to activated pulmonary endothelium prevents metastasis

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ABSTRACT

Enhanced levels of the inflammatory chemokine CCL2 are known to correlate with increased tumorigenesis and metastases, and thereby poor prognosis for cancer patients. The CCL2-CCR2 chemokine axis was shown to facilitate the metastatic initiation through the recruitment of inflammatory monocytes and the activation of endothelial cells at metastatic sites. Both steps are required for efficient cancer cell trans-endothelial migration and seeding in the targeted tissue. The translation of preclinical evidence proved to be challenging due to systemic effects of chemokine inhibition and limited target specificity. Here we tested an approach of a targeted delivery of the CCR2 antagonist Teijin Compound 1 to metastatic sites. VCAM-1 binding peptide tagged liposomes carrying the CCR2 antagonist enabled a specific delivery to cancer cell-activated endothelium. The subsequent binding of target-sensitive liposomes triggered the release of the Teijin Compound 1 and thereby local inhibition of CCR2 in the lungs. Blocking of CCR2 resulted in reduced induction of the lungs vascular permeability, and thereby reduced tumor cell extravasation. However, the recruitment of inflammatory monocytes to the pre-metastatic lungs remained unaltered. Endothelial VCAM-1 targeted delivery of the CCR2 antagonist resulted in inhibition of pulmonary metastases both in a murine (MC-38GFP cells) and a human xenograft (patient-derived cells) model. Thus, timely- and spatially-defined inhibition of CCR2 signaling represents a potential therapeutic approach for treatment of metastasis without affecting homeostatic functions.

1. Introduction

Chemokines are chemotactic cytokines that induce directed migration of cells to maintain homeostasis or to facilitate inflammatory processes. While the primary role of inflammatory chemokines is the recruitment of leukocytes to sites of inflammation, they also contribute to a variety of chronic inflammatory diseases and cancer. Inflammatory chemokines regulate a plethora of pathways involved in cancer progression and metastasis including: leukocyte recruitment, angiogenesis, survival and invasiveness of cancer cells, immune suppression, and cancer cell extravasation [reviewed in 1]. Particularly, higher levels of CCL2 have been linked to cancer progression, metastasis and poor cancer patient survival for several cancer types [2,3]. In animal models, the CCL2-CCR2 chemokine axis was shown to have two essential roles during metastatic initiations: 1) recruitment of inflammatory monocytes to the pre-metastatic niche [4–6] and 2) activation of endothelial CCR2 required for induction of vascular permeability [6].

Metastatic seeding of tumor cells in distant organs is usually associated with enhanced endothelial activation at the site of tumor cells [7]. Entrapped cancer cells in the vasculature have been shown to activate endothelial cells that express several activation markers, including VCAM-1 [7–9]. Furthermore, tumor cell extravasation mostly happens at these sites [8].

While inflammatory chemokines have been considered to be promising therapeutic targets, their targeting revealed to be more difficult partly due to different chemokine involvement in specific diseases. For instance, in rheumatoid arthritis CCR2 has been identified to be a wrong target and rather CCR1 should be used as a target relevant for this condition [10,11]. There is abundant preclinical evidence that CCL2-CCR2-axis targeting significantly affects cancer progression [4–6, 12,13]. Accordingly, neutralization of CCL2 was shown to be effective in preventing metastasis in numerous mouse models of cancer [*e.g.* 4,5]. However, recently it has been demonstrated that cessation of anti-CCL2 antibody treatment can lead to an increased metastatic burden in an orthotopic mammary tumor model using 4T1 cells [14]. The absence or

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the long-term inhibition of the CCL2-CCR2-axis significantly interferes with the homeostatic physiological processes, particularly the egress of inflammatory monocytes from the bone marrow [15,16]. Thus, a targeted and timely defined inhibition of the chemokine signaling in the pre-metastatic niche would be highly beneficial to prevent metastatic initiation without any interference with homeostatic processes of CCL2-CCR2 signaling.

To specifically inhibit CCL2 signaling at metastatic sites, we proposed to use a liposomal-carrier based approach to transport the CCR2 antagonist Teijin Compound 1 (TC1) to sites where cancer cells can activate the lung endothelium. TC1 was designed to inhibit binding of human CCL2 to CCR2b and to block the downstream signaling [17]. Nevertheless, the amino acids required for TC1 binding to CCR2 are conserved between human and mouse sequences [18]. VCAM-1 binding peptide tagged target-sensitive liposomes were previously shown to bind to activated endothelium of atherosclerotic aortas [19]. Here we demonstrate that VCAM-1 targeted delivery of liposomes loaded with the CCR2 antagonist TC1 to metastatic sites in the lungs reduces vascular permeability and prevents generation of pulmonary metastases.

2. Materials and methods

2.1. Cell lines

Patient-derived tumor material was injected in female immune-deficient NSG mice. A single cell suspension of *in vivo* passaged cells was obtained by tissue dissociation of xenograft tumors (P26) using Gentle-MACS (Miltenyi Biotec). The following cell lines were isolated and tested: human melanoma cells Mel_1956, colon carcinoma cells Co_9978, sarcoma cells Sar_10133 and mammary carcinoma cells Ma_4296. Mouse colon carcinoma cells, MC-38 stably expressing GFP (MC-38GFP), were grown as described previously [20]. Human endothelial cells, EA.hy926 line (ATCC) were grown in 10% FCS/DMEM medium as described previously [21]. Human monocytic cell line, U937 cells (a kind gift from Prof. S.C. Silverstein, Columbia University, New York, N.Y., USA), were grown in suspension in 5% FCS/RPMI culture medium.

2.2. Trans-endothelial migration assay

Mouse pulmonary endothelial cells were isolated from lungs using a positive immuno-magnetic anti-CD31-antibody selection and cultivated in DMEM medium supplemented with 20% FCS and with endothelial growth supplement as described previously [6]. Two weeks after isolation the primary endothelial cells were seeded on Boyden chamber inserts (8 μm pores, BD) and cultured until confluency. MC-38GFP cells were added into inserts with or without $CD115^+$ bone-marrow derived monocytes (isolated by positive immune-magnetic selection using anti-CD115-biotin (eBioscience)) in 3% FCS/RPMI medium. Transmigration was stimulated with 10% FCS/RPMI in the bottom of the Boyden chamber in the absence or presence of a CCR2 antagonist = TC1 (20 μg/ml) or CCR5 antagonist = DAPTA (200 μg/ml), both inhibitors were obtained from R&D, and evaluated after 16 h. Transmigrated MC-38GFP cells were counted on the lower side of the insert.

Human endothelial EA.hy926 cells were grown on Boyden chamber inserts (8 μm pores, BD) until confluency. Patient-derived melanoma cells Mel_1956 were added on the confluent endothelial cell layer with or without U937 monocytic cells in 1% FCS/DMEM/F12 medium. Transmigration was stimulated with 10% FCS/DMEM/F12 in the bottom of the chamber in absence or presence of TC1 (20 μg/ml) or DAPTA (200 μg/μl).

2.3. Liposomes

The VCAM-1 binding peptide, sequence NH_2-VHPKQHRGGSKGC-CO_2 [22], was coupled to target-sensitive liposomes (TSL) composed of DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOPA (1,2-dioleoyl-sn-glycero-3-phosphatidic acid), Mal-PEG-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000]) as described previously [19]. Briefly, the phospholipids in chloroform were combined at the ratio of 65:35:2:3 mol% and dried in a rotary evaporator. Then, the lipid film was hydrated with PBS (empty TSL) or with a solution of CCR2 antagonist TC1 (TC1-TSL) at a drug: lipid ratio of 1:5, to reach the final lipid concentration of 10 mM. The resulting multilamellar vesicles were extruded through 100 nm polycarbonate membranes using a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL/USA). The VCAM-1 binding peptide, sequence NH2-VHPKQHRGGSKGC-COOH [22], was coupled at the surface of TSL by sulfhydryl-maleimide chemistry as previously described [19]. To separate the uncoupled peptide and the non-incorporated TC1 from TSL, the Amicon centrifugal filter units with cut-off 100 kDa (Millipore) were used. Rhodamine-PE was added in a 1 mol% ratio to the liposome preparations to stain the TSL or non-TSL (liposomes without VCAM-1 binding peptide).

2.4. Liposome targeting to activated endothelium in vivo

All animal experiments were performed according to the guidelines of the Swiss Animal Protection Law, and approved by Veterinary Office of Kanton Zurich. Mice ($n = 3$) were intravenously injected (i.v.) with 3×10^5 MC-38GFP cells. After 16 h, lungs were perfused through cardiac injection with 2 μmol TSL-Rhodamine-PE or 2 μmol non-TSL-Rhodamine-PE in 5 ml PBS. After 15 min incubation, lungs were washed with PBS, fixed with 3% paraformaldehyde/PBS and embedded in OCT (Tissue-Tek). Cryosections (8 μm) were stained with anti-VCAM-1 antibody (BD), goat-anti-rat IgG (H + L) Alexa Fluor 680 (Invitrogen), and counterstained with DAPI. Stained sections were analyzed with a Leica SP5 confocal microscope.

2.5. Experimental metastasis

Mice were intravenously treated either with 70 μg TC1 in 150 μl 5% glucose/H_2O, 150 μl TC1-TSL (0.79 μmol lipid encapsulating 52 μg TC1), or 150 μl empty-TSL (0.79 μmol lipid) one hour before and 16 h after i.v. injection of 3×10^5 MC-38GFP cells. Mice were terminated after four weeks. Lungs were perfused with PBS and the number of metastatic foci per lung was determined macroscopically.

Human melanoma cells Mel_1956 (5×10^5) were i.v. injected into the tail vein of each mouse. Mice were i.v. treated with 4 mg TC1/kg (corresponding to 80 μg/20 g mouse) in free or liposomal form 4 h before and at the time of tumor cell injection, followed by further injections of the same dose at 4 and 24 h after tumor cell injection. Empty-TSL was injected at a corresponding lipid dose at the same time. After 14 days mice were terminated, macroscopically inspected by autopsy and the number of metastatic foci was counted.

2.6. Flow cytometry analysis of peripheral blood

Blood samples (80 μl) were taken sublingually, diluted with 2 ml of 5 mM EDTA in PBS (EDTA/PBS), and centrifuged for 5 min at 500 g. To lyse erythrocytes, cells were resuspended in 1 ml PharmLyse (BD) and incubated for 15 min at room temperature. Cells were centrifuged for 5 min at 500 g and resuspended in FACS buffer (10 mM EDTA, 2% FCS, in PBS) and stained with CD11b-APC-Cy7, Ly6G-PerCP-Cy5.5, Ly6C-FITC, CD8-PE-Cy7 (all from BD) for 30 min on ice. The cells were washed once with 1 ml PBS and then acquired with a FACSCanto (BD) machine. The results were analyzed by FlowJo software (Tree star).

2.7. Flow cytometry analysis of lungs

Mice were treated with 70 μg TC1 in 150 μl 5% glucose/H_2O, 150 μl TC1-TSL (0.79 μmol lipid encapsulating 52 μg TC1), or 150 μl empty-TSL (0.79 μmol lipid) 10 min prior to i.v. injection of 3×10^5 MC-38GFP cells. After 12 h, lungs were perfused with PBS and digested for

1 h with collagenase A and collagenase D (each 2 mg/ml, Roche) in 2% FCS/RPMI1640 at 37 °C. Cells were filtered through a 100 μm cell strainer (BD) and stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies) followed by staining with: anti-CD45-PE-Cy7, anti-CD11b-APC, anti-Ly6G-PerCP-Cy5.5, anti-Ly6C-FITC antibodies. Data were acquired in the presence of CountBright absolute counting beads (Life Technologies) with a FACSCanto machine (BD) and analyzed by FlowJo software (Tree star).

2.8. Vascular permeability assay (Evans blue assay)

Mice were treated with 150 μl TC1-TSL (0.79 μmol lipid encapsulating 52 μg TC1), or 150 μl empty-TSL (0.79 μmol lipid) 10 min prior to i.v. injection of 3×10^5 MC-38GFP cells. After 24 h mice were intravenously injected with 2 mg of Evans blue in 100 μl PBS. The lungs of mice were perfused 30 min later, pictures taken and homogenized in PBS. The Evans blue dye was extracted with formamide at 60 °C for 18 h. The amount of extravasated Evans blue was determined by spectrophotometry (absorbance at 620 nm).

2.9. Cytometric bead array determination of CCL2

The concentration of hCCL2 in cell-culture supernatants of human cancer cells was determined with the CBA Human MCP-1 Flex Set (BD) according to the manufacturer's instructions. Briefly, 50 μl of pure or 1:10 diluted cell culture supernatants were incubated with hCCL2 capture beads and hCCL2-PE detection reagent (total reaction volume of 150 μl) for three hours at room temperature, and washed once with 1 ml of the supplied Wash Buffer. The beads were reconstituted with 200 μl Wash Buffer and then acquired with a FACSCanto (BD) machine. The mean fluorescent intensity (MFI) of the sample-beads was used to calculate the concentration of hCCL2 in the samples according to the standard-curve of hCCL2.

2.10. Statistical analysis

Statistical analysis was performed with the GraphPad Prism software (version 5.0). Data are presented as mean ± SEM and data with more than 2 groups were analyzed using ANOVA test with Bonferroni's multiple comparison, unless stated otherwise.

3. Results

3.1. TC1 inhibits cancer cell trans-endothelial migration

The CCR2 antagonist Teijin Compound 1 (TC1) was developed to target human CCR2 activation and consequently, to inhibit the ensuing cell chemotaxis induced by CCL2 [23]. However, human and mouse CCR2 have the same amino acid sequence required for TC1 binding [18]. To validate the capacity of TC1 to inhibit murine CCR2, we used the trans-endothelial tumor cell migration experiment employing primary pulmonary endothelial cells. We used mouse colon carcinoma cell line MC-38GFP, which was previously shown to produce CCL2 that is critical for metastatic seeding [6]. The addition of $CD115^+$ monocytes accelerated the transmigration of MC-38GFP cells compared to tumor cells without monocytes. We found a strong inhibition of tumor cell migration in the presence of TC1 (Fig. 1). However, the addition of DAPTA, a CCR5 antagonist, did not affect trans-endothelial migration of MC-38GFP cells. These data confirm that TC1 can inhibit CCR2 in a murine model.

3.2. VCAM-1 binding peptide enables efficient targeting of liposomes to activated endothelium

We hypothesized that the low molecular mass (476 Da) of TC1 would cause fast clearance from circulation and thereby prevent an inhibitory effect on CCR2-based processes *in vivo*. Therefore, we prepared

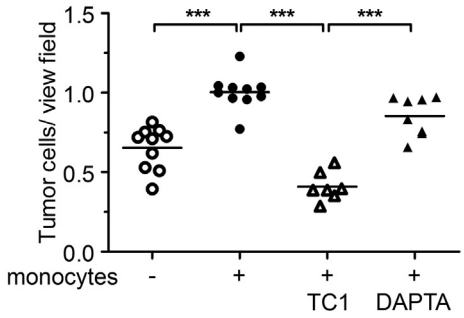

Fig. 1. Transmigration of tumor cells across primary murine pulmonary endothelial cells is inhibited by TC1. Trans-endothelial migration of MC-38GFP tumor cells through endothelial monolayers in the presence or in the absence of monocytes. CCR2 inhibitor TC1 (20 μg/ml) and CCR5 inhibitor DAPTA (200 μg/ml) were added to the co-culture. Transmigrated tumor cells were counted per view field and are presented in relative values. Pooled data from three individual experiments are presented ***, $P < 0.001$.

target-sensitive liposomes (TSL) coupled to a VCAM-1 binding peptide and loaded them with TC1 (TC1-TSL). The binding and TC1 release characteristics of VCAM-1 binding liposomes have been recently described [19]. VCAM-1 was detected on activated endothelium during metastasis [7,9]. To test the specific targeting in a metastatic mouse model, we perfused mice, which received i.v. injection of MC-38GFP cells 16 h earlier, with Rhodamine-PE labeled TSL or non-TSL (without VCAM-1 binding peptide). VCAM-1 expression in the pulmonary vasculature has been detected between 12 and 18 h post-tumor cell injections [7]. Indeed, the presence of VCAM-1 binding peptide on the liposomes ensured strong binding of the TSL to the vasculature in the proximity of MC-38GFP cells, while binding of non-TSL to the activated endothelium could not be detected (Fig. 2). We observed no uptake of TSL in the lungs of control mice, while accumulation of TSL has been detected in the liver after 2 h (data not shown). These data support the specificity of the VCAM-1 binding peptide for the activated endothelium and the ability to selectively target liposomes to the activated vasculature *in vivo*.

3.3. TC1-TSL inhibit pulmonary seeding of metastatic cancer cells

CCR2-mediated interactions have been shown to be required for initiation of metastasis, particularly in the lung tissues [4–6,24]. To test whether TC1-TSL targeting to the pre-metastatic niche affect metastatic seeding, we used a lung experimental metastasis model with MC-38GFP cells. We aimed to inhibit CCR2 signaling during the whole time when tumor cells are in circulation [6], thus it does not completely mimic the clinical situation. Mice receiving TC1-TSL had significantly reduced metastasis when compared to control mice receiving no treatment or mice treated with TC1 loaded non-TSL (Fig. 3). As expected mice treatment with soluble TC1 had no effect on metastasis. However, empty-TSL still had a significant reduction in the number of metastasis when compared to control mice. This is likely due to VCAM-1-directed TSL occupation of activated endothelium, which could impair monocyte function. While monocytes in circulation are required for metastasis [5,6], their reduction significantly affects metastasis [25,26]. Nevertheless, targeted delivery of TC1 using TC1-TSL significantly reduced the incidence of metastasis when compared to empty-TSL (Fig. 3B), which further supports the role of CCR2 during metastatic seeding to the lungs.

3.4. Liposome treatment reduces circulating inflammatory monocytes but their recruitment to metastatic lungs remains unaffected

To test whether liposome treatment affects monocytic population in the circulation, we analyzed peripheral blood of treated mice 24 h after MC-38GFP cell injection. Both liposome preparations TC1-TSL and empty-TSL respectively, equally reduced the number of circulating $Ly6C^{hi}$ inflammatory monocytes when compared to control mice

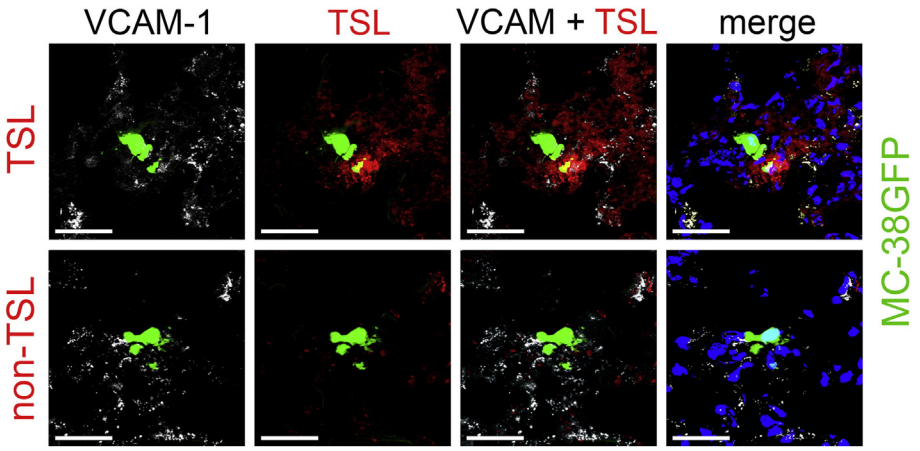

Fig. 2. VCAM-1 binding peptide facilitates efficient targeting of TSL to activated endothelium in lungs. Mice received intravenous injection of MC-38GFP cells 16 h prior perfusion with either Rhodamine-PE-TSL or Rhodamine-PE-non-TSL. Red: liposomes; white: VCAM-1 staining; green: MC-38GFP cells, blue: DAPI. Bar = 30 μm.

(Fig. 4A). TC1 treatment alone had no effect on the presence of Ly6C^{hi} inflammatory monocytes in circulation. These data indicate that TSL have an intrinsic activity leading to partial depletion of circulating monocytes, which requires further investigations.

The presence of cancer cells in the lungs was shown to induce immediate recruitment of inflammatory monocytes (Ly6C^{hi}) to the metastatic microenvironment, which promotes tumor cell extravasation and metastasis [5,6]. Next we assessed whether liposome treatment affects the leukocyte recruitment to the lungs of MC-38GFP injected mice. As expected intravenous injection of MC-38GFP cells induced rapid increase of Ly6C^{hi} inflammatory monocytes in the lung tissue compared to lungs of naive mice (Fig. 4B). But, mice treated with empty-TSL, TC1-TSL, or TC1 alone have shown a similar increase in numbers of Ly6C^{hi} inflammatory monocytes in the lungs. These results indicate that TC1-TSL treatment did not significantly impair the global recruitment of monocytic cells to the early metastatic lungs and thus reduces metastasis by acting locally at metastatic sites.

3.5. TC1-TSL treatment reduces lung vascular permeability in tumor cell injected mice

Previously, we have shown that inhibition of CCR2 affects the tumor cell extravasation in the lungs, which is partially mediated by endothelial CCR2 signaling [6]. Since we observed no reduction of inflammatory monocytes in the metastatic lungs of treated and control mice, we tested whether lung vascular permeability is impaired in TC1-TSL treated mice. Mice injected with TC1-TSL or empty-TSL, and MC-38GFP cells were intravenously injected with Evans blue 24 h later. We detected a

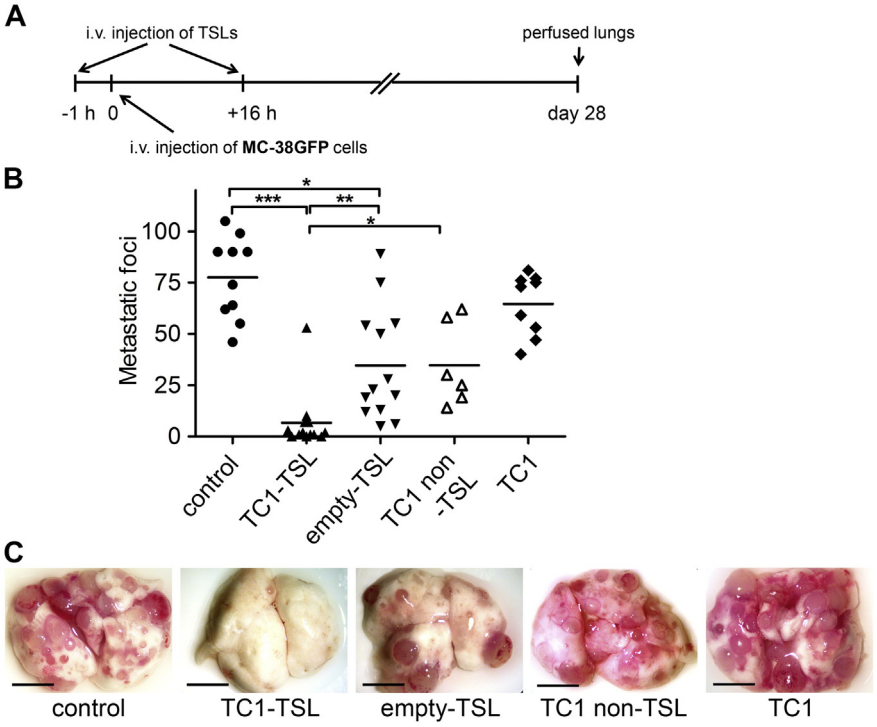

Fig. 3. TC1-TSL attenuates experimental pulmonary metastasis. A) Mice were treated 1 h before and 16 h after i.v. MC-38GFP injection with TC1-TSL, empty-TSL, TC1 non-TSL or free TC1. B) Four weeks later, mice were terminated and metastatic foci were counted macroscopically. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. C) Representative pictures of metastatic lungs. Bar = 5 mm.

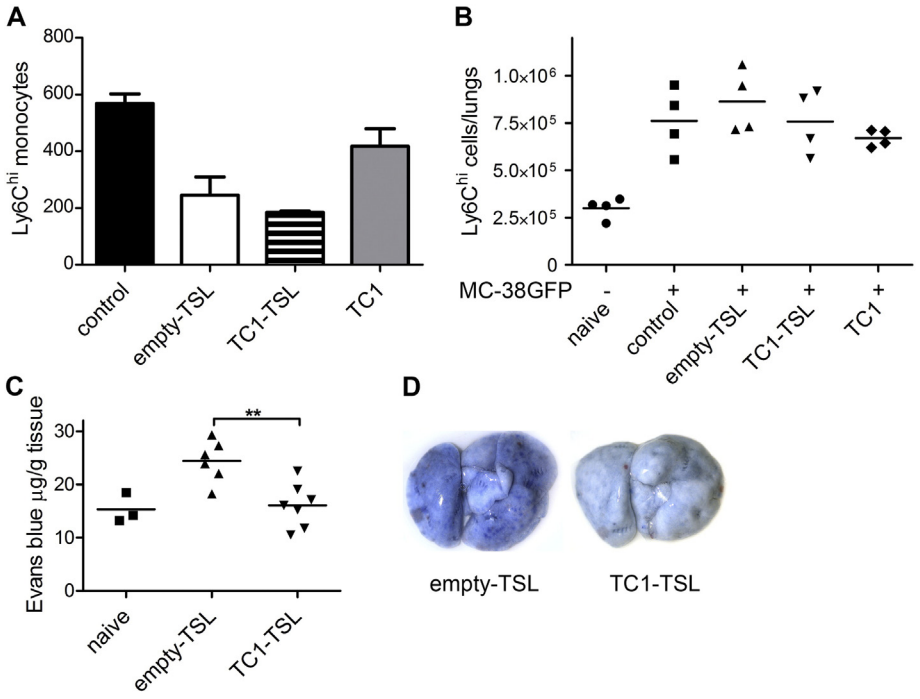

Fig. 4. Liposome treatment affects inflammatory monocytes and lungs vascular leakiness. A) Blood was taken from control mice or mice treated with empty-TSL, TC1-TSL, or TC1 20 h after MC-38GFP injection (4 h after the second liposome injection) and analyzed by flow cytometry. The $CD11b^+Ly6C^{hi}$ cells were standardized to the number of $CD8^+$ cells (1′000 cells). B) Control mice or mice treated with empty-TSL, TC1-TSL, or TC1 were terminated 12 h after MC-38GFP injection. The leukocyte composition in the perfused lungs was analyzed by flow cytometry and the effective number of inflammatory monocytes ($CD45^+CD11b^+Ly6C^{hi}Ly6G^-$) per lungs is presented. C) Empty-TSL- or TC1-TSL-treated mice were intravenously injected with Evans blue 24 h after tumor cell injection. Naive mice (without tumor cell injection) were injected with Evans blue and used as a control. The amount of extracted Evans blue from lung tissue was measured by spectrophotometry. Data were analyzed using Mann–Whitney test **, $P < 0.01$. D) Representative macroscopic images of lungs from Evans blue injected mice.

significant reduction in vascular permeability of TC1-TSL treated mice when compared to empty-TSL injected mice (Fig. 4C). These data are in agreement with the detection of TSL in the proximity of tumor cells and the concomitant endothelial activation (Fig. 2). They further indicate that the local release of TC1 is sufficient for blocking tumor cell extravasation, and thereby the metastatic initiation.

3.6. TC1 inhibits trans-endothelial migration of patient-derived tumor cells expressing CCL2

Increased levels of CCL2 chemokine have been associated with a poor outcome and shorter disease-free survival due to high incidence of metastasis in patients with breast, colon, prostate, and cervical cancer [1]. We tested the CCL2 release of four different tumor cells derived from cancer patients. We detected different CCL2 levels varying from 13.6 to 7181 pg/ml depending on cancer type (Table 1). These data correspond well with previously detected CCL2 levels in the serum of breast and colon cancer patients [2,3].

Next we tested the capacity of TC1 to inhibit trans-endothelial migration of patient-derived tumor cells using the Boyden chamber assay. We selected melanoma cells Mel_1956, which produce significant but not too high amounts of CCL2, thus we can block its activity.

Table 1
CCL2 protein levels detected in medium of cancer cells derived from patients. The cells were grown in cell culture for two days and the protein amount of CCL2 in the medium was determined using the CBA assay. Average (±SD) of at least 3 independent samples is presented.

Patients derived cancer cells	hCCL2 (pg/ml)
Colorectal cancer cells_9978	25.7 ± 1.3
Mammary cancer cells_4296	13.6 ± 6.5
Sarcoma cells_10133	7881.7 ± 2467.6
Melanoma cells_1956	95.9 ± 32.7

While monocytes potentiated trans-endothelial migration of Mel_1956 cells, TC1 clearly inhibited this process (Fig. 5A). The presence of the CCR5 inhibitor DAPTA had no impact on Mel_1956 cell trans-endothelial migration. These data confirm that inhibition of CCR2 impedes the efficient trans-endothelial migration of human tumor cells as was shown also for the mouse cell line MC-38GFP (Fig. 1).

3.7. TC1-TSL inhibit pulmonary seeding of metastatic human cancer cells

To test whether CCR2 inhibition also affects the engraftment of human cancer cells *in vivo*, we used human melanoma cells Mel_1956 derived from a patient in an immune-deficient mouse model. Mice were sacrificed and macroscopically inspected two weeks after i.v. injection of Mel_1956 cells. First we evaluated the incidence of metastasis. In the control group, 9 of 10 mice developed metastases, while the treatment with TC1 reduced that number to 6 of 8 (75%). The treatment with liposomes further reduced the number of metastases per mouse as well as the number of mice found with metastases to 4 of 8 (50%) and 1 of 8 (12.5%) for mice treated with empty-TSL and TC1-TSL, respectively. The number of metastases found in the TC1-TSL treated group was significantly reduced in comparison to the control group (Fig. 5B), while empty-TSL or TC1 alone did not reduce the number of metastases significantly. These data support the finding that TC1 targeting with TSL reduces metastasis also in a xenograft model.

4. Discussion

Metastases are responsible for about 90% of all cancer related fatalities [27] and the lungs are one of the organs affected in many cancers. Although initiation of metastasis can occur early during tumorigenesis, there is evidence that the surgical removal of the primary tumor and the associated inflammation correlates with enhanced metastasis [28]. Furthermore, the surgery is associated with an increase in circulating

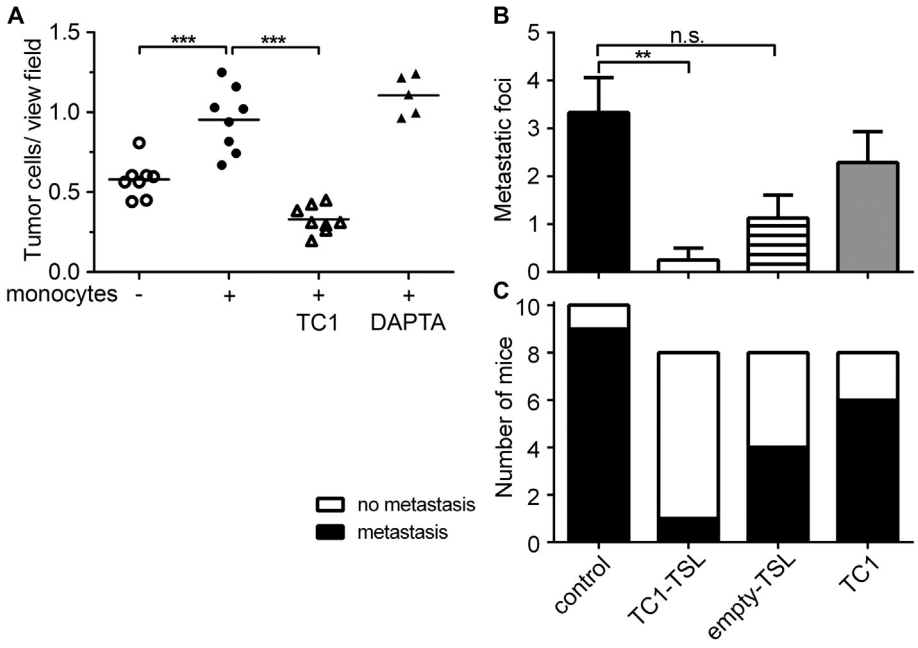

Fig. 5. TC1 inhibits trans-endothelial migration and metastasis of human melanoma cells. A) Migration of human Mel_1956 melanoma cells across human endothelial monolayers in the presence or in the absence of U937 monocytic cells. CCR2 inhibitor TC1 (20 μg/ml) and CCR5 inhibitor DAPTA (200 μg/ml) were added to the assay. Transmigrated tumor cells were counted per view field after 16 h and are presented in relative values. ***, $P < 0.001$. B) Female NSG mice were i.v. injected with Mel_1956 cells. Mice were treated 4 h before, at the time of tumor cell injection, and 4 and 24 h after tumor cell injection with TC1, TC1-TSL, empty-TSL or PBS (control). Mice were terminated after 14 days and metastatic foci were counted. ** = $P < 0.01$; n.s. = not significant. Data are presented as mean ± SEM. C) Number of mice per group with (black) and without (white) metastasis.

tumor cells, which enhances their potential to form metastatic lesion. Tumor cells in the vasculature are highly vulnerable yet accessible for any treatment, and therapeutic efficiency is expected to be the highest during this stage [29,30]. Therefore an adjuvant treatment aiming at potential metastatic tumor cells prior to extravasation may improve the long-term cancer therapy. The mechanism of metastatic initiation is strongly linked to the activation of the CCL2-CCR2 chemokine axis [4–6]. Apparently CCR2-mediated signaling is involved at two levels: 1) recruitment of inflammatory monocytes to the pre-metastatic niche and 2) activation of endothelial CCR2 and thereby the induction of vascular permeability. We expect that the inhibition of the CCL2-CCR2 signaling axis during metastatic initiation will inhibit tumor cell extravasation and subsequently metastases. We provided evidence that the monocyte-mediated trans-endothelial migration of both murine colorectal cancer cells and human melanoma cells *in vitro* is specifically inhibited by the CCR2 antagonist TC1. These results support the functional role of CCR2 signaling in the cross-talk among tumor cells, monocytes, and endothelial cells in this process. Since CCL2-CCR2 signaling has no effect on the recruitment of monocytes in this *in vitro* system, we speculate that CCR2 inhibition predominantly affects endothelial cells. This is supported by previous findings that endothelial- rather than monocytic-CCR2 expression is required for the tumor cell trans-endothelial migration [6]. While the CCR2-mediated recruitment of inflammatory monocytes clearly contributes to metastatic initiation [5,6], we reason that endothelial CCR2 might be the main regulator of vascular permeability induction and cancer cell extravasation [6,24]. This hypothesis is supported by the fact that the TC1-TSL treatment did not reduce the recruitment of inflammatory monocytes to the lungs of MC-38GFP injected mice, yet clearly attenuated metastasis. Importantly, the induction of pulmonary vascular permeability was reduced in TC1-TSL compared to empty-TSL treated cancer cell injected mice. These results indicate that local interference in the lung CCR2 signaling is sufficient for attenuation of metastasis.

The idea of targeted drug delivery to desired tissues is not new. However, the application of a specific liposomal composition, which is triggered by target-binding for controlled destabilization and thus drug release, has not been applied for anti-metastatic approaches. We have used TSL as the carrier for delivering TC1 to sites of tumor cell-activated endothelium. TSL binding to VCAM-1 expressed on the activated endothelium destabilizes liposomes and releases their cargo [19,31]. We showed that TSL-targeting to tumor cell-activated pulmonary endothelium was successful which is in line with previous observations where TSL targeting to the atherosclerotic plaques, expressing VCAM-1, has been performed [19]. Administration of TSL however, caused a depletion of circulating inflammatory monocytes, which was independent of the TC1 presence. Depletion of circulating inflammatory monocytes by clodronate-loaded liposomes was previously shown to severely affect pulmonary seeding of tumor cells [25]. It is thus likely that the liposome composition is partially responsible for this phenomenon, and consequently for a direct or indirect reduction of experimental metastatic foci numbers in both groups (empty-TSL and TC1-TSL) when compared to controls. Nevertheless, TC1-TSL significantly reduced metastatic seeding when compared to empty-TSL, indicating that the targeted delivery of CCR2 antagonist to the metastatic tissue (lungs) almost abolished generation of metastases.

Tumor cell extravasation and thereby seeding of distant tissues happens at sites of the activated endothelium [8]. Our targeted approach confirms that selective inhibition of CCR2 signaling at these sites of metastatic initiation prevents generation of metastases. Contrary, systemic inhibition of the CCR2 signaling in the same time frame did not have any effect on the generation of metastases (TC1 treated mice). This is likely also due to the faster clearance of the compound. Collectively these results show that the temporally and spatially defined inhibition of CCR2 signaling can prevent the generation of metastasis by mainly interfering with induction of pulmonary vascular permeability required for cancer cell extravasation [6,24].

CCL2 has been previously targeted in various pathophysiological models, *e.g.* metastasis and liver damage [5,32]. Antibody-mediated depletion of CCL2 led to reduced infiltration of macrophages to metastatic sites [5], while the use of pharmacological inhibitor to CCL2 reduced macrophages-dependent angiogenesis during liver fibrosis [32]. However, a long-term neutralization of CCL2 by antibody treatment caused a significant depletion of circulating monocytes, which was primarily due to the inhibition of monocyte egress from the bone marrow [14].

A timely defined CCR2 inhibition is unlikely to interfere with global homeostatic roles of CCL2-CCR2 signaling, which affects the levels of circulating inflammatory monocytes [15]. The presented treatment strategy enables an increased therapeutic efficiency due to presence of a high local concentration of the inhibitor at required sites, while keeping other tissues unaffected. However, the further development of alternative nanocarriers would be required for evaluation beyond animal models.

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